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INTRODUCTION

The mechanisms underlying the development and progression of prostate cancer are poorly understood. In addition there are few markers available for the diagnosis of this disease. In an attempt to shed light on the mechanisms of prostate cancer progression and identify novel markers, I am focusing my research efforts on the Rho effector protein kinase N (PKN). This protein kinase has been implicated in prostate cancer: levels of PKN protein are upregulated in prostate cancer, PKN binds the androgen receptor (a key protein in prostate cancer progression), and promotes androgen receptor-dependent transcription (1). The specific aims of my project are to investigate the involvement of PKN family proteins in prostate cancer, and to identify and characterize novel components in the PKN signaling pathway, which may play a role in prostate tumorigenesis. To fulfill these aims I am using small inhibitory RNAs (siRNA) to knock down expression of PKN and the closely-related protein kinase C-related kinase 2 (PRK2) in cell lines and looking at the effects on cellular processes involved in tumorigenesis. In addition I am using the fruit fly *Drosophila melanogaster* as a tool to identify new proteins in the PKN signaling pathway, since the fly has a well-conserved Pkn gene (see figure 1) and many signaling pathways operating in cancer are conserved between flies and humans (2,3).

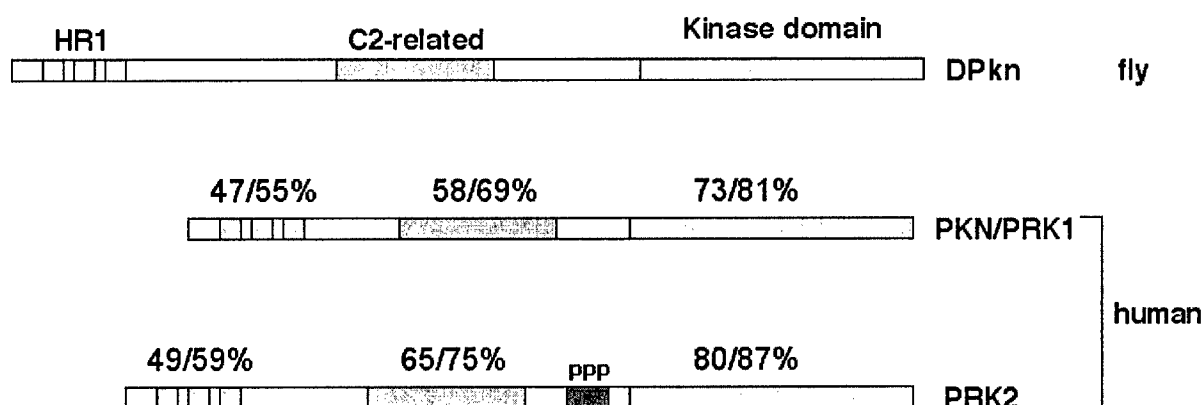


Figure 1. Domain structure of PKN family proteins. Percentage identity/similarity between fly and human proteins is shown above the domains. The HR1 domain mediates binding to Rho and Rac small GTPases. The C2-related domain shows some similarity to the C2 domain of PKCs.

BODY

Task 1. To characterize the role of PKN family proteins in prostate cancer

Previous studies have indicated that PKN is expressed in prostate cancer cells (1). To confirm this and check that PRK2 is also expressed, I obtained two commercially available antibodies: a mouse anti-PKN antibody (Transduction Labs) and a rabbit anti-PRK2 antibody (Cell Signaling). Both antibodies recognized single bands of the expected sizes in PC3 cells, confirming expression of PKN and PRK2 in these cells (data not shown).

I decided to use lentiviral vectors to introduce small hairpin RNAs (shRNA) against PKN and PRK2 into prostate cancer cell lines. To this end I established a collaboration with the laboratory of William Hahn at the Dana Faber Cancer Institute. They cloned five 21 base pair sequences from human PKN and 5 from human PRK2 into the lentiviral pLKO.1ps vector. When these plasmids are introduced into mammalian cells, the sequences are expressed as shRNAs, which can substantially decrease the levels of targeted proteins. As an initial test these vectors were transfected into HEK293 cells and the ability to knock down PKN and PRK2 in HEK293 cells was assayed (see figure 2). Lysates were made from the cells, run on a gel and probed for PKN and PRK2. As obvious from the gel, construct 2 specifically knocks down PKN and construct 25 specifically knocks down PRK2. These can now be packaged into lentiviruses and transfected into prostate cancer cells to generate cell lines in which PKN and PRK2 are stably downregulated. The effect of downregulation on various cellular processes related to tumorigenesis, including proliferation, invasion and migration, will be investigated.

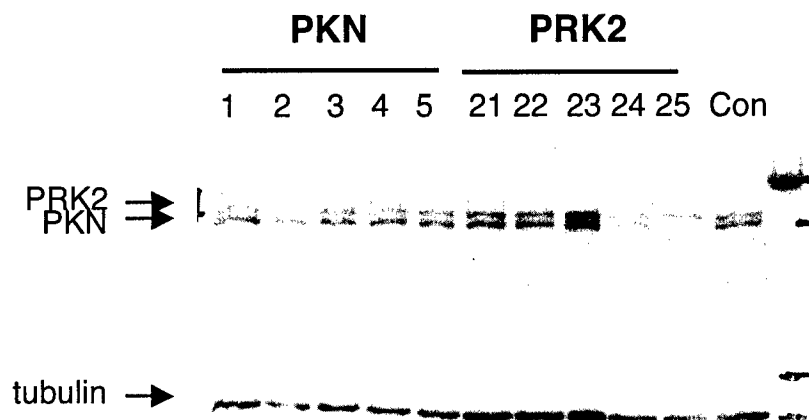


Figure 2. ShRNA-mediated knock down of PKN and PRK2 in HEK 293. Constructs 1 to 5 contained PKN sequences where as constructs 21 to 25 contained PRK2 sequences. Empty vector was used as a control (Con).

Task 2. To identify novel components of the Rho-Pkn signaling pathway by undertaking a genetic screen in Drosophila

I proposed to undertake a dominant modifier screen in *Drosophila* to identify novel components of the Rho-Pkn signaling pathway. In brief, this involves overexpressing a gene of interest (in this case Pkn) in a tissue (such as the wing or eye) where it produces a visible, non-lethal phenotype and then to screen for mutations, which suppress or enhance the phenotype. This technique has been used successfully in the lab to identify novel components of signaling pathways (4, 5).

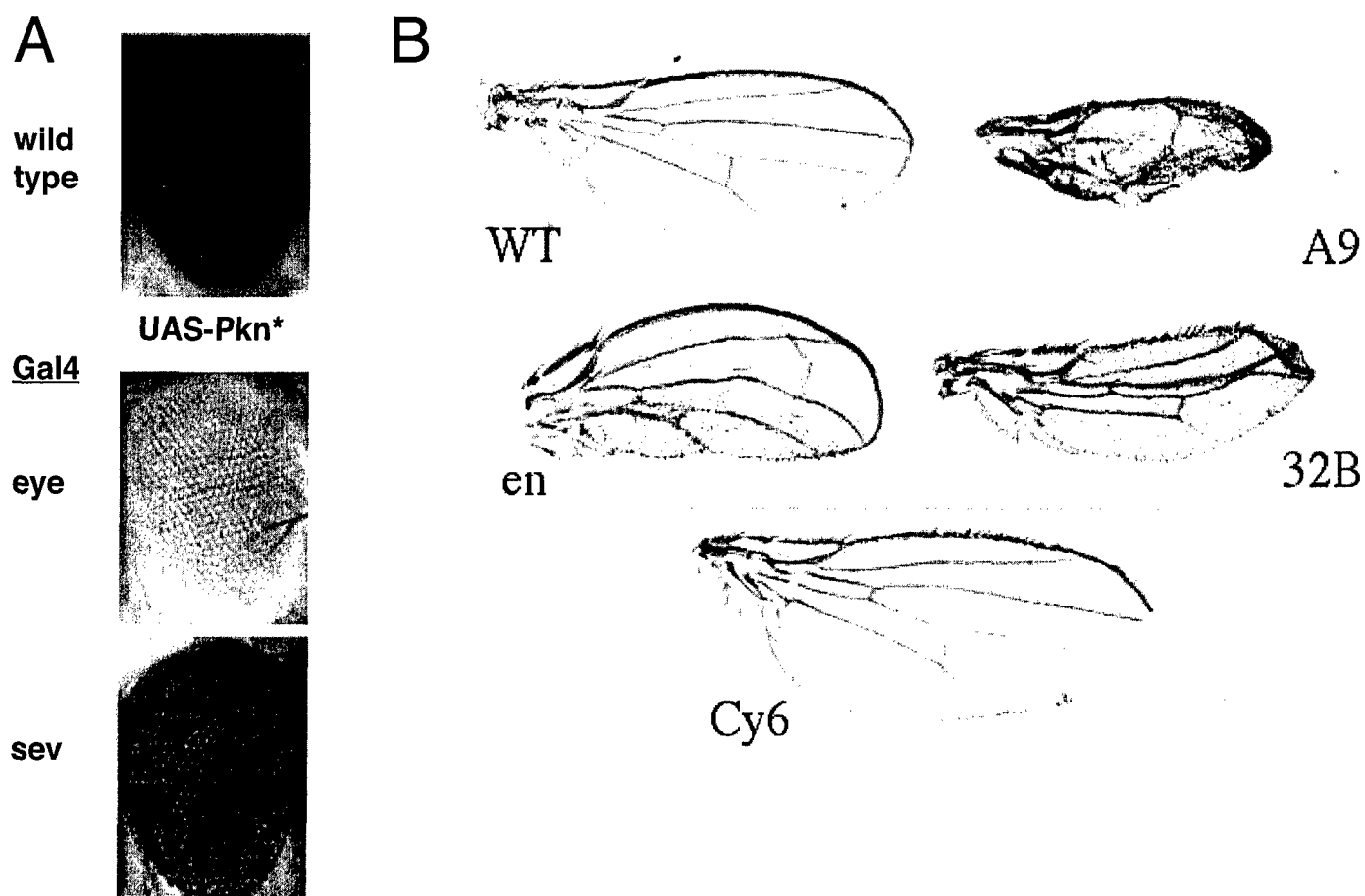
The first step was to generate a suitable phenotype for screening. The UAS/Gal4 system has been used extensively in *Drosophila* to drive expression of a gene of interest in particular tissue (6). I found that driving expression of full length Pkn in the wing or eye using this system did not produce a visible phenotype. This was unsurprising since full length mammalian PKN has been shown to exist in an inactive folded conformation. Therefore, I cloned the kinase domain of Pkn alone, which has been shown in mammalian system to be constitutively active, into the UAS vector to generate UAS-Pkn*. Expression of this domain in the eye using the Sevenless and Eyeless GAL4 produced a mild rough eye phenotype. Driving expression of the Pkn kinase domain in the wing gave phenotypes of varying severity depending on the driver used. These phenotypes included multiple wing hairs and wing blistering and shriveling and are summarized in table 1. Similar wing and eye phenotypes were observed with two different UAS-Pkn* lines. Figure 3a shows representative images of the wings and eyes overexpressing Pkn*.

Driver	Tissue	UAS-Pkn*	UAS-PKC53E*
Cy6	wing margin	missing wing material	missing wing material in some cases
32B	wing, eye	shriveled, planar polarity defects, mwh	ectopic vein material
71B	wing	abnormal hairs	no phenotype
A9	wing	shriveled and blistered, planar polarity defects, mwh	ND
En	wing posterior	buckling, planar polarity defects, mwh in posterior half	ectopic vein material (not fully penetrant)
Act88F	wing (IFM)	no phenotype	ectopic vein material
VE	wing vein	no phenotype	no phenotype
Sev	eye	mild rough eye	no phenotype
Eyeless	eye	mild rough eye	no phenotype
GMR	eye	rough eye	rough eye

Table 1. Phenotypes induced by overexpression of the Pkn and PKC53E kinase domain.

To confirm that the phenotypes generated were specific to Pkn* and not purely the consequence of expressing a kinase domain in the wing and eye, the kinase domain of PKC53E was also cloned into the UAS vector to generate UAS-PKC53E*. PKC53E is a classical protein kinase C and has a kinase domain closely related to that of Pkn (65 % similar). Overexpression of

PKC53* in the eye produced little or no phenotype. However, in the wing it produced very different phenotypes to Pkn*: in general, extra wing vein material (see Table 1 and figure 3).



C

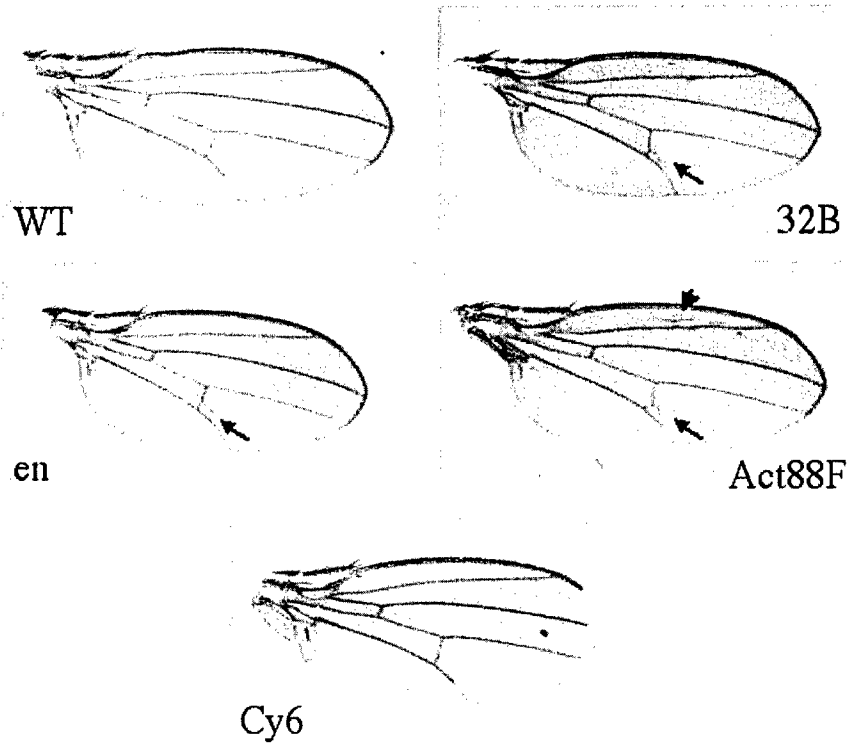


Figure 3. A) Rough eye phenotypes induced by driving expression of UAS-Pkn* in the eye. B) Phenotypes induced by driving expression of UAS-Pkn* in the wing. C) Phenotypes induced by driving expression of UAS-PKC53E* in the wing. Arrows indicate extra vein material.

The phenotype generated using the Engrailed-Gal4 (En-GAL4) driver seemed the most consistent of the Pkn* phenotypes observed. I recombined en-Gal4 and UAS-Pkn* onto the same chromosome and made a stock. Unfortunately I discovered that this UAS/Gal4 combination caused substantial pupal lethality and so was not suitable for generating the large number of adult flies that are necessary for screening. In parallel I had also generated lines expressing the Pkn kinase domain directly under control of the GMR promoter, which drives expression of the Pkn protein in the eye. I found that one copy of GMR-Pkn* gave no phenotype but two copies in cis gave a mild rough eye which was enhanced with three copies (Figure 4). Stocks for these recombinants were viable and fertile and so I decided to use them for screening.

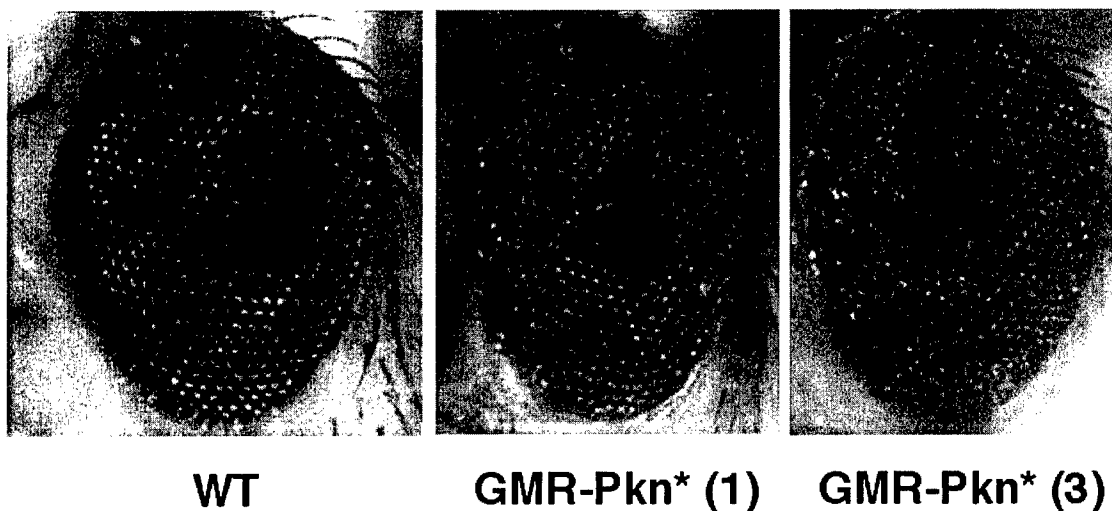


Figure 4. Rough eye phenotypes induced by expression of one copy (GMR-Pkn* (1)) or three copies (GMR-Pkn* (3)) of the Pkn kinase domain.

As a first step in screening, I crossed the GMR-Pkn* flies to the deficiency kits for the 2nd and 3rd chromosomes of *Drosophila*. These are sets of mapped deletions which span 85% of the fly genome. In the first round screen of the deficiency kit for the 2nd chromosome, I identified 6 suppressors and 20 enhancers. The large number of enhancers suggests that these are non-specific. Therefore I will focus on the suppressors. The screen of the 3rd chromosome deficiency kit is still ongoing. I will retest the suppressors I obtained in the screen and also test them against the UAS-Pkn*/en-Gal4 line. Deletions which suppress both the wing and eye phenotype are much more likely to be specific modifiers of Pkn and so represent mutations in components of the Pkn pathway.

KEY RESEARCH ACCOMPLISHMENTS

Lentiviral shRNA constructs generated which knock down levels of PKN and PRK2 protein when introduced into human tissue culture cells

Transgenic flies generated carrying UAS-Pkn*, UAS-PKC53E*, GMR-Pkn* and GMR-PKC53E*

Overexpression of the Pkn kinase domain in wing and eye using the UAS/Gal4 system or the GMR promoter shown to produce phenotypes which can be used for dominant modifier screen

Phenotypes have been demonstrated to be specific for Pkn since overexpression of the PKC53 kinase domain produces different phenotypes

Screen of the deficiency kits with GMR-Pkn* initiated. 20 enhancers and 6 suppressors identified so far on the 2nd chromosome.

REPORTABLE OUTCOMES

Abstracts: "Genetic analysis of Rho effector kinase, PKN, in *Drosophila*" in Program and Abstracts for 45th Annual *Drosophila* Research Conference, Washington DC, March 24-28, 2004

Presentations: Posters at 45th Annual *Drosophila* Research Conference, Washington DC, March 24-28, 2004 and MGH Cancer Center Retreat and Scientific Advisory Board meeting, Woods Hole, October 14th-15th, 2004: "Genetic analysis of Rho effector kinase, PKN, in *Drosophila*"

Infomatics: transgenic flies: UAS-Pkn*, GMR-Pkn*, UAS-PKC53E*, GMR-PKC53E*.

CONCLUSIONS

I have generated and obtained many tools and reagents which I can use for the study of PKN family proteins. I have antibodies which recognize PKN and PRK2 in prostate cancer cells and shRNA lentiviral vectors that specifically knock down PKN and PRK2 in mammalian cells. Thus I am well placed to begin an analysis of the effects of knock down of PKN and PRK2 in prostate cancer cell lines with particular reference to processes involved in tumorigenesis including proliferation, invasion and migration, which may provide novel insights into the mechanisms underlying prostate cancer development.

In terms of identifying new components of the Pkn signaling pathway, I have generated transgenic fly lines overexpressing Pkn with wing and eye phenotypes which can be used for genetic screens. I have already identified suppressors and enhancers of the Pkn eye phenotype in deficiency kit screens. Any modifiers which retest with the eye phenotype will then be tested against the wing phenotype. Then smaller deficiencies spanning the region identified will be tested to narrow down the relevant region. In case the genetic approach is unsuccessful I plan to use the tandem affinity purification (TAP) approach to identify novel Pkn binding partners in *Drosophila* tissue culture cells (7). The *in vivo* significance to Pkn signaling of any binding proteins identified can then be verified by looking for genetic interactions with Pkn. A combination of these approaches should lead to further understanding of a poorly characterized signaling pathway in *Drosophila* and humans which may be relevant to the treatment and diagnosis of prostate cancer.

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